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Seasonal dynamics and pesticide impact on gut microbiome in *Amynthas gracilis* earthworms: A comparative study across agricultural landscapes assessed by 16S rRNA amplicon-based sequencing

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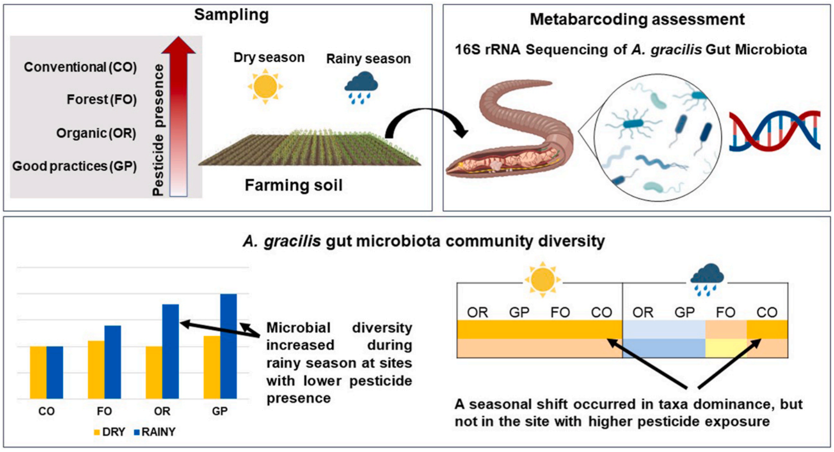
HIGHLIGHTS

- Pesticide use alters *Amynthas gracilis* earthworms gut microbiome.
- Conventionally managed soils showed reduced microbiome diversity, negatively correlated with higher pesticide presence.
- Seasonal shifts in microbiome composition observed.
- Earthworms gut microbiome serve as a bioindicator for pesticide pollution and biodiversity changes in soil.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of pesticides in agriculture can affect the biodiversity of soil ecosystems and interfere with the ecological services they provide. The gut microbiome of earthworms serves as a bioindicator for changes in diversity within one of the most representative groups of soil macrofauna. In this study, we defined a gradient of soil and pesticide use regimes within a horticultural area, including conventional management, good agricultural practices, organic production and forest. Earthworms (*A. gracilis*) from these four conditions were sampled, and their microbiomes were assessed by sequencing the V4 region from the 16S rRNA gene. Comparison of ASVs indicated the presence of 142 bacterial genera among all the samples, with Actinobacteriota, Proteobacteria, Firmicutes and Bacteroidota being dominant phyla. Simpson's diversity index revealed diminished biodiversity in the gut microbiomes of earthworms from conventionally managed soil, correlating negatively with the presence of the insecticide

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chlorpyrifos. Furthermore, a seasonal shift in the dominance of bacterial taxa was observed between dry and rainy seasons. These shifts were evident in the gut microbiome of organisms from organically managed and good practices soils but not in the conventional site. Our results demonstrate that earthworm's gut microbiome serves as a responsive bioindicator for biodiversity changes in agricultural landscapes, suggesting that key features of the microbial community can be altered by pesticide exposure.

1. Introduction

Biodiversity and its associated ecosystem services are essential for agricultural landscapes, improving soil quality and providing ecological stability (Erisman et al., 2016; Kooch et al., 2025). Despite the acknowledged effectiveness of pesticides in pest management and crop protection (Özkara et al., 2016), the use of such substances represents a risk for soil ecosystems under agricultural use, or even remote areas receiving residues from crop applications (Brühl et al., 2024; Gunstone et al., 2021). There is ample research indicating loss of biodiversity in agricultural areas and beyond (Albaseer et al., 2025; Beketov et al., 2013; Emmerson et al., 2016; Kumar et al., 2021) due to the effects of pesticides, demonstrating their role on the degradation of such ecosystems.

Earthworms are key elements of soil biodiversity, contributing to nutrient cycling and soil structure enhancement. Earthworms activity leads to an increase in the availability of vital nutrients such as nitrogen and phosphorus, crucial for plant growth (Chauhan, 2014; Lejoly et al., 2024), and their presence fosters microbial diversity, thereby augmenting soil fertility (Bhadauria and Saxena, 2009).

Several studies have shown that the gut environment of earthworms harbors distinct microbial communities compared to surrounding soil (Horn et al., 2003; Yang et al., 2024). This may explain the pivotal role that gut microbiome plays in organic matter decomposition dynamics and nutrient cycling (Medina-Sauza et al., 2019), as well as in the degradation of different contaminants, including organic pollutants, heavy metals, and microplastics (Sun et al., 2020). It is well known that environmental characteristics and food sources can affect the composition of earthworm's gut microbiota (Sapkota et al., 2020), and exposure to pesticide-contaminated soil has been shown to disrupt the structure and abundance of earthworm's gut microbiomes (Astaykina et al., 2022), with several studies reporting shifts in the gut microbiome composition, including increases in some bacterial genera and decreases in others (Krishnaswamy et al., 2021; Owagboriaye et al., 2021). Furthermore, the gut microbiota forms a complex relationship with earthworms and plays a crucial role in their physiological functions, immune system and ability to digest food (Liu et al., 2018), as well as their ability to adapt to new environments (Yang et al., 2023). For this reason, exposure to pesticides could have a negative effect on worm populations, impairing their ecological function.

Consequently, earthworm's gut microbiome has become a good bioindicator, related to the fitness and functions of their host, and responding to different chemical environmental pollutants (Sun et al., 2020). Currently the application of metagenomic analysis is used to assess the microbial community composition in earthworm's gut and their changes due to external stressors (Owagboriaye et al., 2021; Xia et al., 2022). Such approach has demonstrated how exposure to pesticides with different biocide actions, including herbicides, fungicides and insecticides, can affect the abundance and diversity of bacteria in the gut of different earthworm species (Chang et al., 2021; Song et al., 2022; G. Zhu et al., 2021).

As a case to study effects of pesticide pollution on soil biota, Costa Rican agriculture is recognized for its high use of pesticides, approximately 34.5 kg of active ingredient per hectare per year (Vargas, 2022). In the northern part of the country, the area of Zarcero sustains an intensive horticultural production, including conventional, organic, and good agricultural practices models (Raman et al., 2022). In this setting, considerable pesticide use has been diagnosed; as well as their residues in the environment, and their adverse environmental effects (Raman et al., 2022; Weiss et al., 2023). In this regard, recent evidence demonstrated the vulnerability of earthworms to pesticide exposure in the Zarcero area (Brenes-Bravo et al., 2025). Evasive behavior and alterations in biomarkers indicative of biotransformation and neurotoxic processes were observed in resident *Amynthas gracilis* individuals as response to soils highly exposed to pesticides. Such responses evidence how earthworms are sensitive to this type of pollution, in a real scenario.

Considering the responsiveness of earthworm's gut microbiomes as a bioindicators and the intensive pesticide use in Zarcero's horticulture, in this study we utilized DNA metabarcoding to investigate the gut microbiome of *A. gracilis* earthworms inhabiting four sites characterized by varying levels of pesticide contamination. We hypothesized that any discernible correlation between soil pesticide concentrations and shifts in the earthworms' gut microbiome could potentially influence the health of the organisms and thereby impact their ecological functionality. Our main goal was to ascertain whether the presence of pesticides in soil correlates with structural changes in the intestinal microbiome of these organisms.

2. Materials and methods

2.1. Study area

The study was carried out in a horticultural area of Zarcero, Costa Rica. Three farms with different management regimes were selected: conventional production with high pesticide use (CO), production with good agricultural practices and reduced use of pesticides (GP) and organic production (OR). A fourth site on the edge of a forested area (FO) was included as soil with no agricultural use. A more detailed characterization of the site locations, soil types, physical-chemical properties and pesticide presence is described in (Brenes-Bravo et al. (2025). Briefly, that study indicated that soils from Conventional production CO, GP and OR, possess a predominantly loamy texture, while the forest soils (FO) soil exhibited a sandy texture. This characterization was similar for moisture and organic matter, with these parameters showing a better condition in CO, GP and OR, while FO soil showed lower amounts of organic matter and moisture. These differences were observed in both dry and rainy season. The pH in all soils was slightly acidic, with GP soil showing the lowest pH value in both seasons. Regarding the presence of pesticides, the highest number of pesticides in the dry season and especially in the rainy season were recorded in CO soils, where the count was the highest (42 pesticides detected), mainly azole group fungicides and organophosphate insecticides. It is also noteworthy that chlorpyrifos was the only pesticide detected in all soils during the dry season, with the FO soils showing the highest value (71.9 ng/g dw).

2.2. Sample collection

Sampling was conducted in Zarcero, Alajuela, Costa Rica, once during the dry season and once during the rainy season of 2022. A theoretical gradient in pesticide application regime was defined based on land-use type: soils coming from FO site with no agricultural management, OR farming soil managed without agrochemicals, GP farming soil with reduced agrochemical use, incorporating alternative biological strategies such as biofertilizers and crop rotation, and CO farming soil characterized by intensive agrochemical use and mechanical plowing.

At each site, a sampling point was defined within a 50 cm × 50 cm grid, from which, 20 cm of soil depth was removed and sieved to collect the *Amynthas gracilis* earthworms. The individuals collected were adults, identified primarily by the presence of a clitellum, with an average length between 7 and 10 cm. In addition, a 2 kg soil sample was taken for pesticide residues presence and physical-chemical parameter analysis (Brenes-Bravo et al., 2025). On site, the proximal region of the earthworms, extending 5 mm posterior to the clitellum, was dissected, subsequently, the gut contents of each individual were extracted and placed into a 1.5 mL microcentrifuge tube, which was stored in liquid nitrogen during transportation to the laboratory. Soil samples were kept at −20 °C, and earthworms samples were kept at −80 °C until further analysis.

2.3. Total DNA extraction

The DNeasy PowerLyzer PowerSoil Kit (Qiagen Corp.) was used for extracting total microbial DNA from the gut content, following the manufacturer's specifications. The DNA concentration, quality and integrity were verified through gel electrophoresis and NanoDrop 2000 (Thermo Fisher Scientific). Additionally, a PCR was performed on all the samples to assure the integrity of the 16S ribosomal RNA (rRNA) gene, using the universal primers 342F (5′-CTACGGGGGGCAGCAG-3′) and 806R (5′-GGACTACCGGGGTATCT-3′) (Mori et al., 2014). DNA was stored at −20 °C until sequencing was performed.

2.4. Sequencing by metagenome amplicon and bioinformatics analysis

For this study, 40 samples were analyzed: forest soil ($n = 10$), organic farming soil ($n = 10$), farming soil with good practices ($n = 10$) and conventional farming soil ($n = 10$) distributed in each culture systems on rainy ($n = 5$) and dry seasons ($n = 5$). The V4 region from the 16S rRNA gene was PCR amplified using the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) y 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The amplicons were used for library preparation and sequencing with the Illumina Novaseq 6000 system 2 × 250 bp (Novogene Inc.). Cutadapt 3.5 (Martin, 2011) was used to remove primer sequences, and DADA2 (Callahan et al., 2016) was used to infer amplicon sequence variants (ASV) in the R environment v4.3.2 (R Core Team, 2024). A naïve Bayesian classifier (Wang et al., 2007) and the SILVA 138.1 database (Quast et al., 2013) were employed for taxonomic assignment. ASVs assigned to eukaryote, chloroplast, and mitochondria were removed. Packages PICRUSt2 v2.5.2 (Douglas et al., 2020), HMMER (Eddy, 2011), EPA-ng (Barbera et al., 2019) and GAPPA (Czech et al., 2020) were used for the phylogenetic placement of the ASVs in a reference phylogenetic tree from the Integrated Microbial Genomes (IMG) database (Markowitz et al., 2012). ASVs that aligned poorly to the reference alignment were also removed from the analysis. Raw data files are available under BioProject ID PRJNA1134209 from the National Center for Biotechnology (NCBI).

2.5. Statistical analyses

Diversity indices, such as Shannon (1948), Chao1 (Chao, 1987) and Simpson (1949) were obtained with phyloseq v1.42.0 (McMurdie and Holmes, 2013), and Faith's phylogenetic diversity (Faith, 1992) with picante v1.8 (Kembel et al., 2010) after normalizing the count table by scaling with ranked subsampling to the lowest number of reads in a sample (Beule and Karlovsky, 2020). Alpha diversity metrics were summarized as box plots made with ggplot2 version 3.4.3. Normal distribution was assessed with Shapiro-Wilk test, and differences in diversity indices between sites were evaluated with Kruskal-Wallis and post-hoc Dunn test with the Benjamini-Hochberg false discovery rate (FDR) adjustment for multiple pairwise comparisons. In addition, to investigate the relationship between continuous explanatory variables and diversity indices, a Spearman correlation analysis, as well as generalized linear models with a quasi-Poisson distribution, were applied.

For beta diversity analysis, uninformative ASVs were removed with an abundance and prevalence filter step, retaining only ASVs with more than 4 reads in at least 10 % of the samples (Dhariwal et al., 2017; Motiei et al., 2020). Weighted UniFrac distance between samples was calculated and the results were presented as 2D ordination plots based on principal coordinates analysis (PCoA) (Lozupone et al., 2011) using phyloseq v1.42.0. Permutational multivariate analysis of variance (PERMANOVA) awas conducted using the vegan package v2.6-4 (Oksanen et al., 2024), to assess the statistical significance of the patterns in the microbial community composition. Physicochemical variables were plotted as fitted arrows onto the PCoA using the envfit function to assess the association between the variables and the structure of the bacterial community.

ANOVA-like differential expression (ALDEx2) analysis for compositional data was employed to identify specific bacterial taxa with varying relative abundance between sites. In addition, aldex.corr from the package ALDEx2 was used to calculate Pearson, Spearman and Kendall correlations between the taxa and continuous variables. Finally, heatmaps of relative abundance profiles at phylum, class and genus levels were generated using ampvis2 v2.8.7 (Andersen et al., 2018).

3. Results

Bacterial community diversity and structure was characterized in the guts of *A. gracilis* earthworms from soils collected at sites with different regimes of pesticide use. A previous assessment (Brenes-Bravo et al., 2025) described environmental characteristics of the studies sites and identified the site managed as conventional horticulture (CO) as the soil most contaminated with pesticide residues. In this study, we describe how the gut microbial community of *A. gracilis* varies between dry and rainy seasons, and how this seasonal shift was hindered at the most polluted site. Furthermore, during the rainy season, when a greater number of pesticides were detected in CO, earthworms from this site exhibited lower microbiome diversity. Environmental factors like moisture or pH, but also the presence of pesticides can influence such changes in diversity. The results detailed below suggest that pesticide pollution can alter the gut microbiome of earthworms. Based on these findings, we discuss how such modulation may interfere with their fitness and the ecosystem services they provide to the soil.

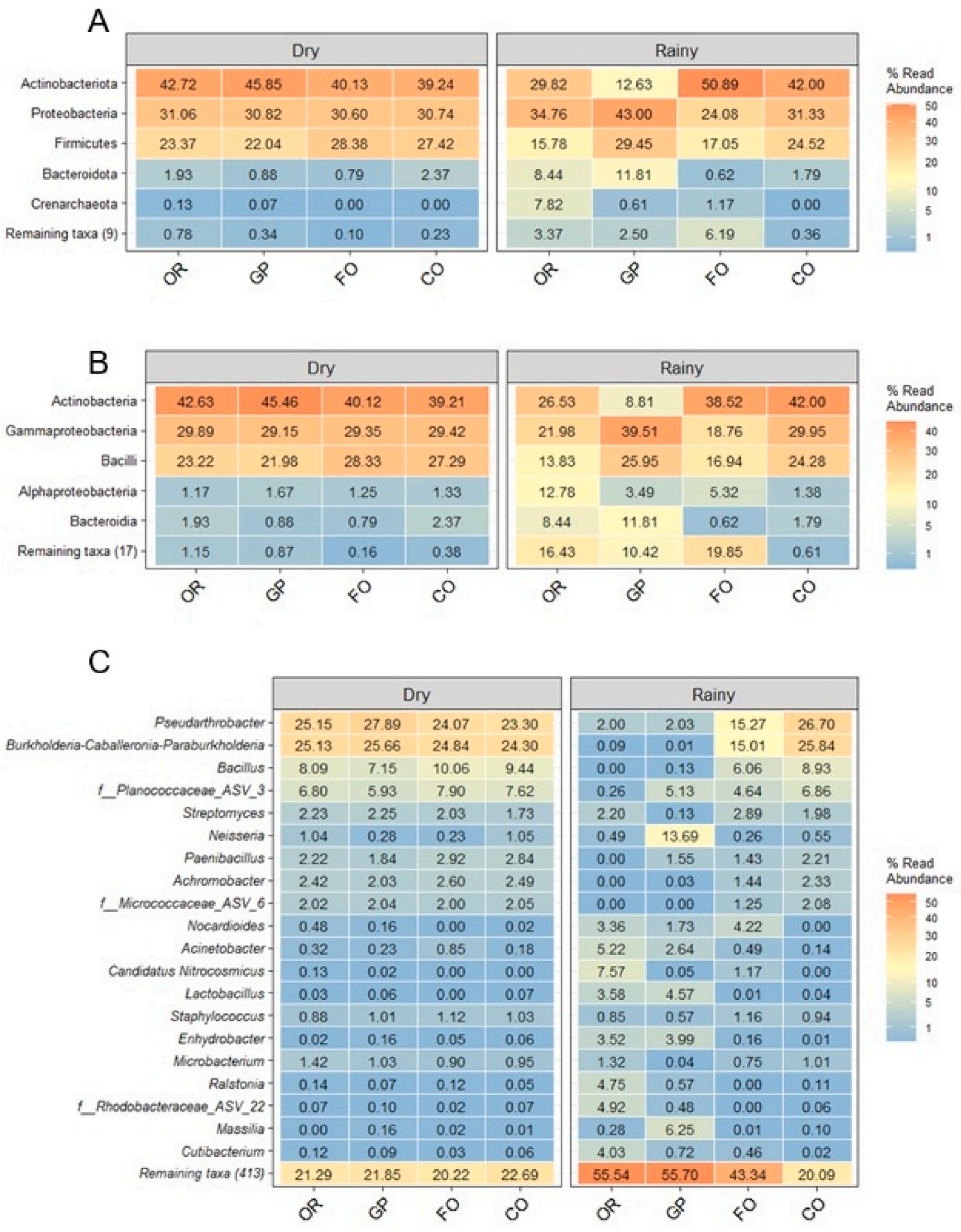
3.1. Sequencing

A total of 4,755,498 raw paired-end reads were produced from the 38 earthworm intestinal samples. Following PCR primer trimming with Cutadapt, and quality trimming, denoising, read merging and chimera filtering with DADA2, 1,921,779 high-quality sequences were kept, with an average of 50,573 reads per sample. DADA2 also yielded a total of 7878 ASVs, of which 4184, 3712, 2596 and 2027 ASVs were present in the OR, GP, FO, and CO samples, respectively.

3.2. Bacterial community composition

The gut microbiome of *A. gracilis* consisted of 14 phyla, 22 classes and 129 genera with assigned taxonomy. Most of the ASVs belonged to the phyla Actinobacteriota (369 ASVs), Proteobacteria (250) and Firmicutes (212). Actinobacteriota was also the most abundant phyla in the gut microbiome of *A. gracilis* in all four sites (Fig. 1A). The phyla Proteobacteria and Firmicutes were also abundant in all four sites, with relative abundances ranging from 15.78 to 43 %. The classes Actinobacteria, Gammaproteobacteria and Bacili were the most abundant (Fig. 1B), while the genera *Pseudarthrobacter*, *Burkholderia-Caballeronia-Paraburkholderia*, and *Bacillus* were the most abundant (Fig. 1C).

According to ALDEx2 analysis, in the dry season 10 genera presented differential abundance between the sites ($p < 0.05$), including some of



A

	Dry OR	Dry GP	Dry FO	Dry CO	Rainy OR	Rainy GP	Rainy FO	Rainy CO
Actinobacteriota	42.72	45.85	40.13	39.24	29.82	12.63	50.89	42.00
Proteobacteria	31.06	30.82	30.60	30.74	34.76	43.00	24.08	31.33
Firmicutes	23.37	22.04	28.38	27.42	15.78	29.45	17.05	24.52
Bacteroidota	1.93	0.88	0.79	2.37	8.44	11.81	0.62	1.79
Crenarchaeota	0.13	0.07	0.00	0.00	7.82	0.61	1.17	0.00
Remaining taxa (9)	0.78	0.34	0.10	0.23	3.37	2.50	6.19	0.36

B

	Dry OR	Dry GP	Dry FO	Dry CO	Rainy OR	Rainy GP	Rainy FO	Rainy CO
Actinobacteria	42.63	45.46	40.12	39.21	26.53	8.81	38.52	42.00
Gammaproteobacteria	29.89	29.15	29.35	29.42	21.98	39.51	18.76	29.95
Bacilli	23.22	21.98	28.33	27.29	13.83	25.95	16.94	24.28
Alphaproteobacteria	1.17	1.67	1.25	1.33	12.78	3.49	5.32	1.38
Bacteroidia	1.93	0.88	0.79	2.37	8.44	11.81	0.62	1.79
Remaining taxa (17)	1.15	0.87	0.16	0.38	16.43	10.42	19.85	0.61

C

	Dry OR	Dry GP	Dry FO	Dry CO	Rainy OR	Rainy GP	Rainy FO	Rainy CO
Pseudarthrobacter	25.15	27.89	24.07	23.30	2.00	2.03	15.27	26.70
Burkholderia-Caballeronia-Paraburkholderia	25.13	25.66	24.84	24.30	0.09	0.01	15.01	25.84
Bacillus	8.09	7.15	10.06	9.44	0.00	0.13	6.06	8.93
f__Planococcaceae_ASV_3	6.80	5.93	7.90	7.62	0.26	5.13	4.64	6.86
Streptomyces	2.23	2.25	2.03	1.73	2.20	0.13	2.89	1.98
Neisseria	1.04	0.28	0.23	1.05	0.49	13.69	0.26	0.55
Paenibacillus	2.22	1.84	2.92	2.84	0.00	1.55	1.43	2.21
Achromobacter	2.42	2.03	2.60	2.49	0.00	0.03	1.44	2.33
f__Micrococcaceae_ASV_6	2.02	2.04	2.00	2.05	0.00	0.00	1.25	2.08
Nocardioides	0.48	0.16	0.00	0.02	3.36	1.73	4.22	0.00
Acinetobacter	0.32	0.23	0.85	0.18	5.22	2.64	0.49	0.14
Candidatus Nitrocosmicus	0.13	0.02	0.00	0.00	7.57	0.05	1.17	0.00
Lactobacillus	0.03	0.06	0.00	0.07	3.58	4.57	0.01	0.04
Staphylococcus	0.88	1.01	1.12	1.03	0.85	0.57	1.16	0.94
Enhydrobacter	0.02	0.16	0.05	0.06	3.52	3.99	0.16	0.01
Microbacterium	1.42	1.03	0.90	0.95	1.32	0.04	0.75	1.01
Ralstonia	0.14	0.07	0.12	0.05	4.75	0.57	0.00	0.11
f__Rhodobacteraceae_ASV_22	0.07	0.10	0.02	0.07	4.92	0.48	0.00	0.06
Massilia	0.00	0.16	0.02	0.01	0.28	6.25	0.01	0.10
Cutibacterium	0.12	0.09	0.03	0.06	4.03	0.72	0.46	0.02
Remaining taxa (413)	21.29	21.85	20.22	22.69	55.54	55.70	43.34	20.09

Fig. 1. Heatmaps of the relative abundance of the 5 phyla (A), top 5 classes (B) and top 20 genera (C) for the microbial community in the 4 sampling sites within the dry and rainy seasons. Organic management (OR), good management (GP), conventional management (CO) and forest (FO).

the more abundant genera, such as *Paenibacillus* and *Achromobacter* (Supplementary Table 1). *Achromobacter* and *Sphingobacterium* relative abundance were the lowest in the GP samples, while *Paenibacillus* and *Fictibacillus* relative abundances were lower in OR and GP samples when compared to FO and CO samples. Interestingly, the genera *Leptotrichia* and *Prevotella* were significantly more abundant in the OR samples than in the other sites.

In the rainy season, 17 genera presented differential abundance between the sites ($p < 0.05$), including some of the most abundant genera, such as *Pseudarthrobacter*, *Burkholderia-Caballeronia-Paraburkholderia*, *Bacillus*, *Streptomyces*, *Paenibacillus*, *Achromobacter*, *Nocardioides*, and *Microbacterium*. The relative abundance of most of these genera was significantly higher in the FO and CO samples than in OR and GP samples.

A succession in the dominant genera was observed between the dry and the rainy seasons according to relative abundances (Fig. 1C). This succession was drastic in OR and GP, where the higher relative abundances for the rainy season shifted to genera that were not dominant during the dry season. This was evident in a decrease in the relative abundance of *Pseudarthrobacter*, *Burkholderia* complex, and *Bacillus*, and an increase in the abundance of genera that were less represented in the dry season, such as *Neisseria*, *Nocardioides*, *Acinetobacter*, *Lactobacillus*, *Enhydrobacter* and *Candidatus* Nitrocosmicus. This seasonal effect was not as drastic in organisms from FO soils as compared with OR or GP; and most interesting, it did not occur at all in the animals from the CO site.

3.3. Correlations between taxa and environmental variables

The only pesticide that showed significant correlation with the relative abundance of bacterial taxa was chlorpyrifos. According to aldex.corr analysis, the phyla Firmicutes, Actinobacteriota and Proteobacteria correlated positively with chlorpyrifos concentration. Contrarily, Chloroflexi correlated negatively with chlorpyrifos. Similarly, the relative abundance of 5 classes (Actinobacteria, Bacilli, Gammaproteobacteria, Alphaproteobacteria, and Bacteroidia) was positively correlated with chlorpyrifos concentration. Meanwhile, the relative abundance of Acidimicrobiia and Thermoleophilia was negatively correlated with chlorpyrifos.

Regarding genera, the relative abundance of 19 taxa (*Rhodococcus*, *Brevibacterium*, *Pseudarthrobacter*, *Promicromonospora*, *Sphingobacterium*, *Fictibacillus*, *Planococcus*, *Planomicrobium*, *Paenibacillus*, *Ensifer*, *Phyllobacterium*, *Achromobacter*, *Burkholderia-Caballeronia-Paraburkholderia*, *Bacillus*, *Stenotrophomonas*, *Microbacterium*, *Streptomyces*, *Micromonospora*, and *Staphylococcus*) were positively correlated with chlorpyrifos concentration. Conversely, the relative abundance of 7 genera (*Nocardioides*, *Mycobacterium*, *Gaiella*, *Solirubrobacter*, *Cutibacterium*, *Candidatus* Nitrocosmicus, and *Phenylobacterium*) were negatively correlated with chlorpyrifos. No correlation between the taxa relative abundance with soil pH, OM, and moisture, as well as with the concentration of other pesticides was found.

3.4. Alpha diversity

Alpha diversity indexes were obtained for each of the samples. A Kruskal-Wallis H test showed that there was no significant difference in Shannon index between samples from the different sites ($\chi2_{(3)} = 5.82$, $p = 0.121$). A similar result was obtained for the estimated Chao1 indices. However, there was a significant difference in Simpson index between samples from the different sites within the rainy season ($\chi2_{(3)} = 8.74$, $p = 0.03$). A post-hoc Dunn's test identified a significantly lower Simpson index in samples from CO when compared to OR and GP (BH-adjusted $p = 0.04$, Fig. 2).

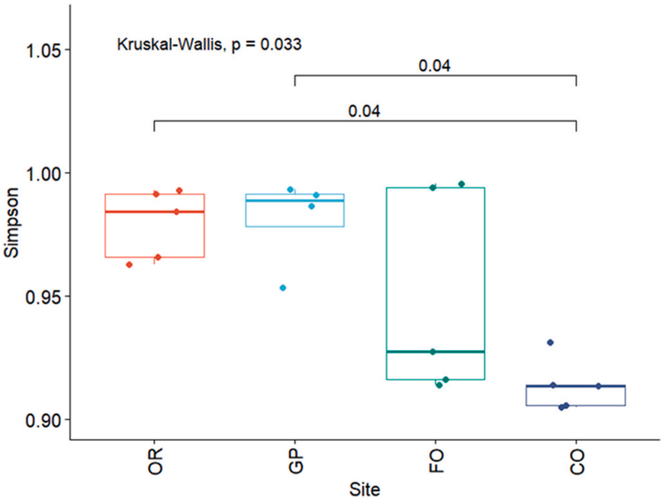


Fig. 2. Bacterial alpha diversity represented as Simpson index of the microbial communities in the 4 sampling sites within the rainy season. Organic management (OR), good management (GP), conventional management (CO) and forest (FO).

No strong correlations were found between Chao1, Simpson and Shannon and pH, OM or moisture, both in dry and rainy seasons. Generalized linear models (GLM) analysis confirmed the lack of effect of pH, OM and moisture over the Shannon index in the dry season, however in the rainy season, OM and moisture had a significant effect on Shannon index ($p < 0.05$) when considered separately, as OM reduces, and moisture increases, Shannon diversity. In addition, in the rainy season, GLM demonstrated a statistically significant influence of the interaction between pH and OM ($p < 0.05$), and pH and moisture ($p < 0.05$) on the Shannon index, however no significant interaction was observed for OM and moisture. The negative impact of pH on Shannon lessens as OM increases, and similarly, the negative effect of OM is weaker at higher pH, suggesting a buffering effect. The same outcome was obtained when fitting a model with an interaction between pH and moisture.

GLM analysis indicated that OM and moisture influenced the Simpson index, both in the dry and rainy seasons. Similarly, GLM indicated a significant influence of the interactions between pH and OM ($p < 0.05$), and pH and moisture ($p < 0.05$) on the Simpson index, both in the dry and rainy seasons, yet again no interaction was observed for OM and moisture.

Shannon index was negatively correlated with chlorpyrifos ($r = -0.55$, $p < 0.05$) but only in the rainy season, and a similar trend was observed for Simpson. In addition, in the rainy season, Simpson also presented a negative correlation with linuron ($r = -0.52$, $p < 0.05$), flutolanil ($r = -0.52$, $p < 0.05$), and pyraclostrobin ($r = -0.52$, $p < 0.05$), and in the dry season presented a positive correlation with azoxystrobin, flutolanil and linuron ($r = 0.63$, $p < 0.01$).

GLM analysis detected that Shannon was only affected by chlorpyrifos ($p < 0.05$), and that Simpson was only affected by azoxystrobin ($p < 0.05$) in the dry season. Similarly, Simpson was affected by chlorpyrifos and flutolanil ($p < 0.05$) in the rainy season.

3.5. Beta diversity

According to the PCoA analysis based on weighted UniFrac distances (Fig. 3), the samples from OR and GP formed distinct clusters along the first axis according to the season, and within the rainy season, most samples from OR clustered tightly together with samples from GP along the second axis. No clear separation of samples within the dry season was detected along the first and second axes. Regarding the microbiome of earthworms from CO, the absence of seasonal variation was clear, agreeing with the observation regarding relative abundance described earlier. PERMANOVA detected significant differences in the structure of the microbial community at different sites (pseudo-F = 1.9423, $R^2 = 0.1463$, $p \leq 0.05$) and seasons (pseudo-F = 9.6599, $R^2 = 0.2116$, $p \leq 0.01$). The interaction was found to be statistically significant (pseudo-F = 4.3204, $R^2 = 0.5020$, $p \leq 0.01$), indicating that the effect of season on community composition varies by site.

4. Discussion

Earthworms make up the biggest component of the animal biomass found in the soil (Yang et al., 2023) and their feeding is mainly related to microorganism. Given that their gut microbiota is richer than that of the soil itself (Bhadauria and Saxena, 2009; Egert et al., 2004), earthworms exert an enriching effect through the deposition of decomposed organic matter with a balanced microbial population (Govindarajan and Prabaharan, 2015). In this regard, changes in gut microbiota communities can adversely impact the fitness of their hosts, affecting behavior, feeding, adaptation, as well as their ecological role by limiting their

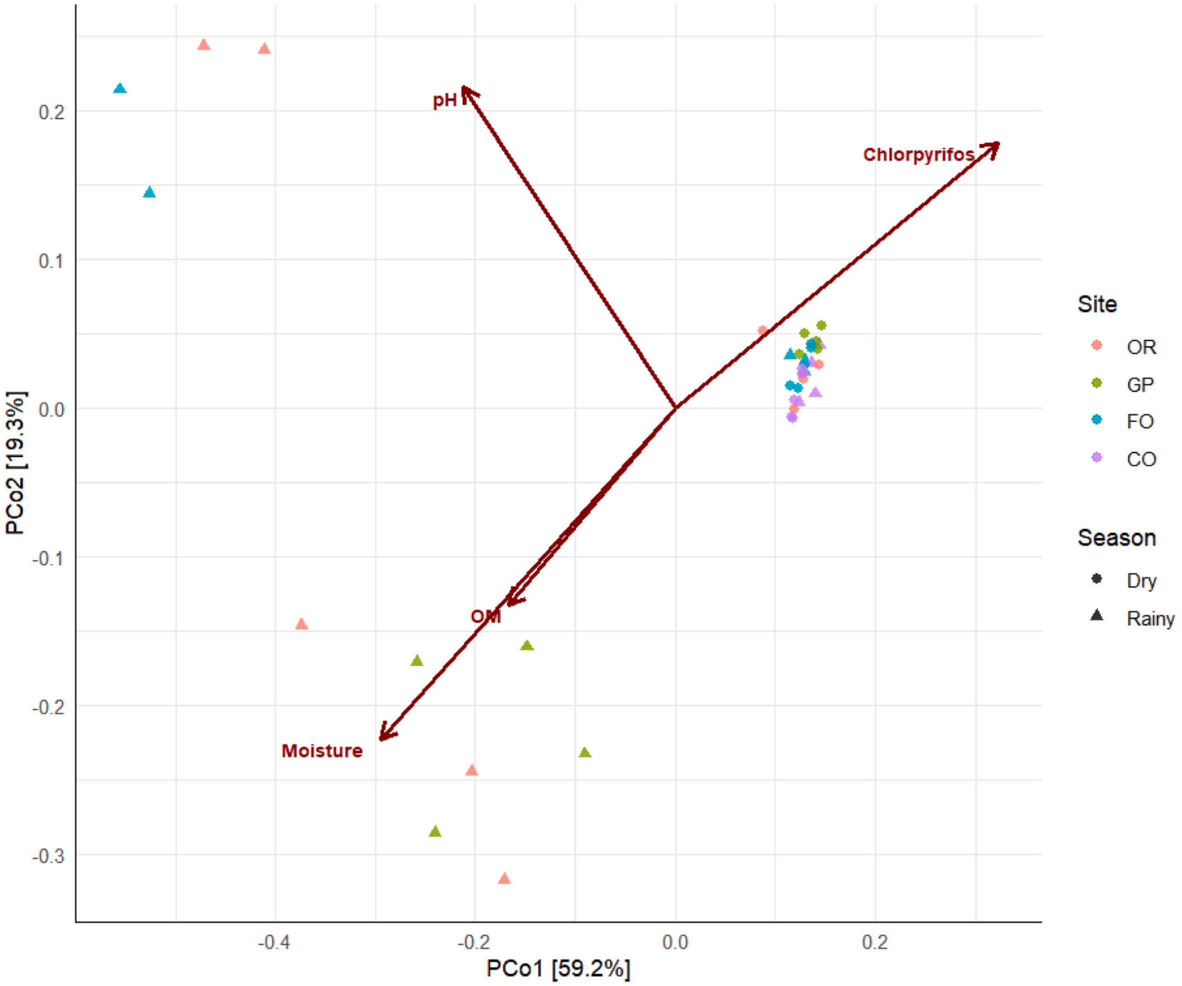


Fig. 3. Principal coordinate analysis (PCoA) based on weighted UniFrac distances from ASV relative abundance data. Distribution of the samples from the organic (OR), good practices (GP), forest (FO) and conventional (CO) sites is shown in the first two axes of the PCoA.

ability to improve soil conditions (Berg et al., 2016).

Changes in the composition of the gut microbiome structure in earthworms can be mediated by the physicochemical conditions of the areas they inhabit, as well as by the availability of different food sources (Liu et al., 2018; Yang et al., 2023). Additionally, the presence of contaminants can play an important role in the specialization of microbiomes, as has been previously observed in soils (Walder et al., 2022). In our research, very similar conditions of pH, organic matter, moisture and texture were observed among the different sites evaluated under agricultural soil conditions (CO, GP and OR), except for forest soil (FO), which reported low amounts of organic matter and moisture. Considering these conditions, our results indicate that the gut bacterial community in *A. gracilis* is primarily composed of Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes in both dry and rainy seasons, in agreement with other studies that reported a similar composition of the microbiome in *E. fetida* and *E. andrei* (Aira et al., 2022; Liu et al., 2018; Ma et al., 2017; Singh et al., 2015). This suggests that the observed microbial community structure in *A. gracilis* could correspond to a common characteristic core for earthworms.

At the genus level, considerable changes were observed in the gut microbiome, mainly mediated by the effect of season. Seasonal changes in the microbial community composition have been reported before on earthworms (Hao et al., 2024), and it is well known that the activity of earthworms is affected by seasons (Singh et al., 2021). Such changes in the microbiome could be linked to seasonal associated variations in environmental conditions (moisture and organic matter). Furthermore, as we know, among microbiome ecological interactions in soil (mutualism, parasitism, commensalism, etc.), competition for resources dominates these interactions (Wang and Kuzyakov, 2024). In this regard, the changes in moisture, pH and organic matter content during the rainy season could improve the conditions for some species to compete, enhancing the diversity of gut microbiome in worms from GP, OR and FO. Furthermore, seasonal shifts in gut microbiome have been described in other organisms, including insects (Ferguson et al., 2018), fish (Savard et al., 2023), or mammals (Baniel et al., 2021; Fan et al., 2022). These studies have associated the plasticity of gut microbiome with the adaptation of the host organisms to changing environmental variables and food sources (Baniel et al., 2021). In the case of earthworms, Drake and Horn (2007) referred to their guts as an anoxic microenvironment which sustains a transient microbial community that benefits from the available ingested organic matter and participates in its fermentation. However, ours is the first report of seasonal shift in gut microbial community in this species. But even more interesting was the absence of a community shift in the guts of earthworms from the conventional agriculture site (CO). Considering that other key variables such as humidity and organic matter content were similar to the other sites where community shifted, we suggest that the higher pesticide pollution present in CO could be associated with this impairment.

4.1. *Changes in the earthworm gut microbiome due to pesticides*

Regarding the relationships between the pesticides detected in the studied sites and the gut microbiome of their earthworms, there was a clear influence of chlorpyrifos on reduced diversity and the absence of a seasonal change observed in the organisms from CO. Krishnaswamy et al. (2021) described how this organophosphate is capable of reducing diversity and changing dominance in the gut microbiome of an earthworm. Furthermore, some authors have reported a considerable decrease in the microbial diversity in earthworms exposed to organophosphate, carbamate and organochlorine pesticides (Chang et al., 2021; Kavitha et al., 2020). This effect has been observed even with the application of pesticides under recommended rate (Astaykina et al., 2022). Explaining this behavior can be complex; however, we know that CO soils had the highest pesticide number detected, especially high in the rainy season. Furthermore, it is known that bacterial groups from the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria have demonstrated a high capacity for degrading carbamate, organophosphate, organochlorine and pyrethroid pesticides (Manish et al., 2021). These results are consistent with our findings and may suggest a specialization of the microbiome in CO caused by the high presence of pesticides.

There are other factors related to the fitness of the *A. gracilis* from the CO site that might explain this outcome. In a parallel study (Brenes-Bravo et al., 2025), we observed inhibition of cholinesterase activity (ChE) and a trend towards a decrease in glutathione S-transferase activity (GST) in individuals of *A. gracilis* sampled from CO soil in the rainy season. We know that the gut microbiota and the host organism's fitness are directly related. Additionally, it is known that neurotoxicity affects their behavior (feeding) (Nofyan et al., 2017; Pavlov et al., 1992). Thus, the organism's feeding activity could be affected by the observed ChE inhibition, indirectly affecting the composition of the organism's gut microbiome. On the other hand, a mechanism proposed by Chang et al. (2021) associates disruption in antioxidant capacity and energy resources with a lag in the organism's growth and metabolism, which could reflect a greater sensitivity of the gut microbiota to soil contaminants. Similar effects have been observed before (Yu et al., 2022) with microplastic exposure, where a relationship between bacterial diversity and variations in antioxidant capacity in *E. fetida* has been demonstrated.

4.2. Functions of the microbiome

Our results have demonstrated a higher relative abundance of the genera *Pseudarthrobacter*, *Burkholderia* complex, *Bacillus*, *Solibacillus*, and *Streptomyces* during the dry season in the gut microbiome of *A. gracilis* from all the assessed sites, maintaining a very stable core in terms of relative abundance.

Pseusdarthrobacter (Actinobacteria) are denitrifying bacteria (Su et al., 2019) so their presence in the earthworm's gut microbiome could play an important role in the nitrogen cycle, being involved in the degradation of nitrogenous compounds. Additionally, organisms identified from the *Burkholderia* complex (Proteobacteria) have been associated with biocontrol functions, bioremediation, and plant growth promotion in soils, as well as pathogenic effects in humans (Rojas-Rojas et al., 2018). While the presence of these organisms has been previously observed in the gut content of *Aporrectodea caliginosa* (Aira et al., 2022), their role in the earthworm's gut is still not well understood.

Bacteria of the genus *Bacillus* (Firmicutes) have been related to improved cellulose digestion capacity (Yang et al., 2023) and are crucial in nutrient cycling and the decomposition of organic matter in the earthworm's gut (Chang et al., 2021). Additionally, *Bacillus* is known to produce phosphoesterase, an enzyme capable of degrading organophosphorus pesticides (Krishnaswamy et al., 2021), which may be related to a degradation function in the organisms sampled from all soils during the dry season, primarily due to the presence of chlorpyrifos in those soils. Furthermore, members of the genus *Solibacillus*, from the family Planococcaceae (Firmicutes) has been previously reported in the gut microbiome of *E. fetida* (Pérez-Pérez et al., 2018). Although its intestinal function has not been described, this bacterium has been observed to participate in the decomposition of organic matter and the formation of humic substances (Zhu et al., 2020). As we know, humic substances improve soil fertility and nutrient absorption (Trevisan et al., 2010), and earthworms are considered "hot spots" for the transformation of these substances (Tikhonov et al., 2016). Likewise, *Streptomyces* (Actinobacteria) was enriched in the gut microbiome of earthworms and is closely related to carbon and nitrogen metabolism (Zhang et al., 2022). An increase in the abundance of this bacterium has been observed in the intestines of earthworms exposed to pesticides, which suggests a link between its increase and the degradation of these compounds (Astaykina et al., 2022).

Regarding the taxa involved in the abrupt shift in microbial community observed in the rainy season, *Nocardioides* (Actinobacteria) is known for having chemoheterotrophic, aerobic chemoheterotrophic, and aromatic organic compounds degradative functions, with high presence in the early phases of composting (Tortosa et al., 2021). It has also been related with the rapid degradation of contaminants, utilizing them as sources of carbon and nitrogen (Ma et al., 2023). Additionally, strains of *Acinetobacter* (Proteobacteria) can utilize a wide range of carbon sources as food (Ren and Palmer, 2023). An increased presence in the gut microbiome has been suggested as a response to cope with the stress in earthworms exposed to cadmium (Zhou et al., 2021). A similar effect has been observed in earthworms exposed to tetracycline (Yin et al., 2021). However, the presence of pesticides in the rainy season in the soils of GP (2 substances), OR (7 substances) and FO (2 substances) was low as compared to the soil from CO site (42 substances) (Brenes-Bravo et al., 2025). Therefore, it is possible that the observed increase in organic matter created good conditions for the proliferation of these bacteria.

In the case of *Lactobacillus* (Firmicutes), its wide distribution in horticultural soils (Raman et al., 2022), such as those analyzed in this study, has been observed, showing biocontrol functions against pathogens harmful to crops. Additionally, this genera is used as probiotics to improve gastrointestinal health (Msimbira et al., 2022). In earthworms fed with *Lactobacillus* probiotics, a considerable increase in size and weight has been observed compared to controls (Sakthika and Anbumalar, 2022). This suggests that their presence has a positive impact on the health of the earthworms.

Finally, it is known that *Candidatus* Nitrocosmicus (Thaumarchaeota) plays a role in the nitrification process, degrading ammonium compounds into nitrite (Sauder et al., 2017). This suggests that the role of *Candidatus* Nitrocosmicus in the earthworms's gut is related to nitrification of ammonium from decomposed organic matter or organic fertilizers in soils, into a bioavailable form of nitrogen, which can be good for improvement of plant growth.

Our results should contribute to the knowledge of the earthworm's gut microbiome, its importance and behavior as a bioindicator. Furthermore, our data describes key features of the response of this microbial community to pesticide pollution, *in situ*. We also noticed that gut microbiome studies have exclusively focused on bacteria, at this point we must suggest that future assessments include eukaryotes, like fungi, protists and even small metazoans being part of the earthworm diet (Sapkota et al., 2020).

5. Conclusions

To our knowledge, this is the first study to analyze the composition of earthworm's gut across different pesticide regimes and seasons in the Central American region. Our results demonstrated significant changes in the gut microbiota of earthworms between seasons and suggest that high pesticide loads can reduce the diversity in these microbial communities. High pesticide exposure can also interfere with the capacity of earthworm's gut microbiome to adapt to seasonal changes, potentially affecting their ecological role in soils.

CRediT authorship contribution statement

Gabriel Brenes-Bravo: Writing – original draft, Methodology, Investigation, Formal analysis. **Frank Solano-Campos:** Writing – original draft, Resources, Investigation, Funding acquisition, Formal analysis, Data curation. **Clemens Ruepert:** Writing – original draft, Resources, Investigation, Funding acquisition, Formal analysis, Data curation. **Freylan Mena:** Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT, an AI language model developed by OpenAI, in order to check the language and review the writing. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2025.144660.

Data availability

Data will be made available on request.

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